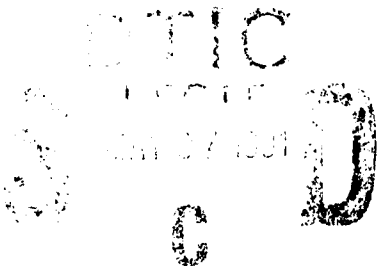
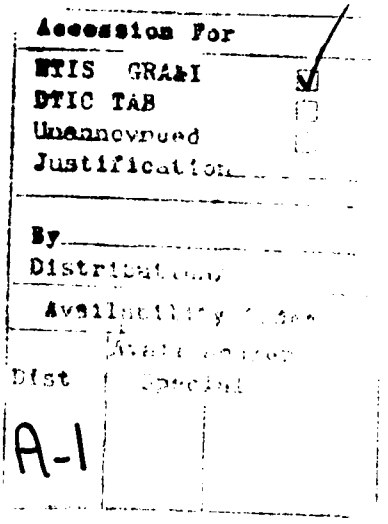


POSSIBLE MECHANISM FOR DENERVATION EFFECT ON WOUND HEALING



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The iron-transport plasma protein transferrin is required for cell proliferation and is present in peripheral nerves. We have tested the hypothesis that release of this factor from axons may be involved in the growth-promoting effect neurons exert on cell proliferation during certain processes of repair in poorly vascularized tissues. Using regeneration in an amphibian as the model system, transferrin was purified, antibodies against it were produced, and the factor was studied qualitatively and quantitatively during nerve and limb regeneration. Immunocytochemistry showed the protein to be present in axons and Schwann cells, as well as in the perineurium of peripheral nerves. An immunoassay developed in the project revealed that the transferrin content of sciatic nerves increases dramatically during regeneration and that the protein is included in the anterograde fast transport component of axons. These results are consistent with the hypothesis that neural release of this factor may be involved in the trophic effect of nerves observed during regeneration.

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SUMMARY

Basic research was performed to study the potential involvement of transferrin, an iron-transport protein required for cell proliferation, in the neural effect on wound healing and tissue regeneration. The system of tissue repair under investigation was the regenerating limb of the axolotl, in which growth is strictly dependent on unknown factors from peripheral nerves. The rationale of the study was to quantify transferrin in segments of ligated normal and regenerating nerves in order to obtain information with which to test the hypothesis that axons transport transferrin to cells of the regenerating tissues.

Before experiments of this nature could be undertaken, axolotl transferrin and antibodies against this factor had to be produced so that immunoassays could be developed to measure this protein in nerves, regenerating limbs, and other tissues from axolotls. These goals were accomplished in the first phase of the project and during the last phase the immunoassay was used to obtain data regarding axonal transport of transferrin. Results indicate that this protein is part of fast anterograde component of axonal transport in regenerating nerves. This is the first demonstration of axonal transport of a general trophic factor required for survival and proliferation of animal cells.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985).

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Body of Report

Statement of the Problem under Study

This contract has supported basic research into the mechanism by which peripheral nerves promote cell proliferation and growth during processes of tissue repair. This trophic effect of nerves has been recognized for many years, particularly in certain injured tissues or organs with reduced vascular supplies, but the molecular basis for the neural influence remains unclear. The medical importance of this problem lies primarily in the frequent failure of injuries and surgical wounds to heal at normal rates in tissues and organs that have experienced severe reduction in the nerve supply (1,2).

The animal model used in these investigations is the urodele amphibian, which has a particularly well-developed capacity for repair and regeneration in nearly all of its organ systems (2). Appendages of such animals are capable of complete regeneration after amputation and the early phase of the regeneration process has been clearly shown to be dependent on an influence from nerves for cell proliferation and growth (2,3). The regenerating amphibian limb is well-suited for investigations of the growth-promoting properties of nerves because the cellular events involved have been carefully characterized histologically (2), because partial or complete denervation of the regenerating tissue is relatively simple to achieve (unlike various commonly used mammalian models of wound healing), and because the effects of partial and complete denervation on proliferative activity

during regeneration have been examined quantitatively in previous studies (3,4).

This project specifically investigated the possibility that neural transferrin may be important in the growth-promoting effect of neurons. Transferrin, a glycoprotein used for iron-transport, is provided to cells throughout the body by circulation in plasma and interstitial fluid, and is required for cell proliferation. The importance of transferrin for growth of animal cells is apparantly due to the iron cofactor requirement of enzymes controlling DNA synthesis (5,6). Observations that peripheral nerves are rich in transferrin (6,7), together with the importance of this factor for survival and proliferation of animal cells, suggested that an analysis of the availability and possible neural transport of transferrin during urodele limb regeneration might provide useful new information on the role of nerves in this developing system.

Background and Review of the Literature

Certain basic features of the neural influence on limb regeneration have been known for many years and are generally accepted (2,3). The trophic effect can be provided by sensory, motor, or autonomic nerves, as well as by central nervous tissue. Nerves are needed for cell proliferation and growth of the regenerating limb (the blastema), but are not necessary for morphogenesis. The effect is mediated by protein factor(s)

present in both central and peripheral nervous tissue that are not species-specific.

Technical problems have hindered attempts to identify and isolate the growth-promoting activity from amphibian nerves. Chief among these have been the relatively small amount of nervous tissue available for protein purification and the lack of a rapid, sensitive bioassay for stimulation of proliferative activity in the regeneration blastema. Primary organ cultures of blastemas have been used in attempts to develop an assay system for factors promoting blastema cell proliferation. We have reviewed results obtained by such an *in vitro* approach (8). Although indicative of certain kinds of factors important for blastemal cell growth, this bioassay has not yet led to the characterization of growth-promoting proteins released from neurons.

Like amphibian limb regeneration, development of skeletal muscle in embryos is also dependent on uncharacterized protein factor from the growing nerve supply (9). Primary cultures of embryonic chick myoblasts have been used in many laboratories as *in vitro* assays to elucidate the biochemical basis for this trophic effect of neurons. This work clearly showed that the addition of extracts of brain or sciatic nerve to culture medium, like co-culture with explanted neural tissue, promotes growth of cultured myoblasts and fusion of these cells into large, multinucleated myotubes which differentiate into muscle fibers (7,9,10).

Using such a bioassay, Oh and Markelonis (7) purified a protein from chicken sciatic nerve that was capable of promoting *in vitro* the entire sequence of muscle formation, from myoblast proliferation to production of muscle fibers. These authors named the purified protein "sciatin", but upon further characterization it was found to be the iron-transport factor transferrin (7).

Further work in several laboratories has shown that transferrin accumulates in growing neurons of both birds and mammals (reviewed in ref. 11). There is evidence that this accumulation may involve both neuronal uptake by a receptor-mediated mechanism (12) and synthesis of the protein by neurons (13). While the physiological and developmental significance of transferrin in peripheral nerve remains to be established, it has been suggested that axonal delivery of iron is necessary for synthesis of respiratory enzymes in mitochondria of neuronal growth cones (11) and enzymes in Schwann cells involved in myelination (14).

In a preliminary investigation of the importance of this factor for proliferation of regeneration blastema cells we found that human serum transferrin stimulated the DNA labeling index, incorporation of ^{3}H-thymidine, and the mitotic index in the organ culture assay (15). While other serum proteins had no effect, addition of transferrin at a concentration of 25 ug/ml to culture medium with reduced serum content (1% fetal bovine serum) caused all three growth parameters to increase to levels normally seen in medium containing 10% serum. The rate of blastema cell

proliferation in medium with 10% serum is similar to that *in vivo* and results in extensive outgrowth of cells from the explant.

Concentrations of transferrin above the optimal dose were found to *inhibit* cell proliferation in cultured blastemas (15), an effect also observed in dose-response studies with mammalian cells (16). It has been suggested that the inhibitory effect on cell proliferation observed *in vitro* at high transferrin concentrations is due to competition for receptors between iron-free transferrin and iron-carrying transferrin, resulting in reduced delivery of iron to the cells (17). In support of this hypothesis, we found that adding $FeCl_3$ to the medium relieved the inhibitory effect of the high transferrin concentration (15). All aspects of our preliminary study, including relief of the inhibitory effect by iron, have been confirmed by another laboratory using a similar but more sensitive bioassay (18).

Using antiserum to plasma transferrin from the salamander *Pleurodeles waltii* which cross-reacted with that of the newt (*Notophthalmus viridescens*), we demonstrated by immunodiffusion the presence of this protein in extracts of newt brain and peripheral nerve (19). That this neural transferrin may promote the blastema cell proliferation seen with brain extracts was indicated by the finding that removal of the iron from such an extract with the chelator desferrioxamine rendered the extract inactive, with full activity restored by the readdition of ferric iron (19). Moreover, the dose-response curve for brain extract was similar to that of transferrin and the inhibition of cell

proliferation at high concentrations of brain extract was also reversed by $FeCl_3$ (19).

These results suggest that transferrin in neural extracts is involved in the stimulatory effect of such extracts on blastemal cell growth. Encouraged by these findings, we undertook the present investigation on the availability and delivery of transferrin in peripheral nerves during the proliferative phase of normal amphibian limb regeneration *in vivo*.

Rationale of the Study

The major technical objectives of this project were (1) to purify transferrin from the urodele amphibian, the axolotl (*Ambystoma mexicanum*), (2) to generate antibodies against this protein, and (3) to develop a sensitive enzyme immunoassay for determining the concentration of transferrin in axolotl tissue extracts. The rationale for this plan was that if significant release of transferrin from nerves occurs in the regenerating limb, then denervation of the limb should lower the transferrin concentration in the blastema, which could be measured by the immunoassay. Similarly, assaying transferrin concentrations in segments at different levels in ligated sciatic nerves should provide evidence regarding the possible axoplasmic transport of this factor.

The availability of antiserum against axolotl transferrin was also expected to allow better immunohistochemical localization of transferrin in axolotl nerves and regenerating

limb tissues than was possible with the heterologous antiserum against Pleurodeles transferrin used in our earlier studies.

Experimental Methods

Purification of axolotl transferrin

Transferrin was purified from axolotl serum using the procedure of Werner et al. (20). Serum was obtained by cardiac puncture of adult animals, 15 to 25 cm in length, anesthetized in 1% benzocaine. After 24 h dialysis against 0.02 M K_2HPO_4, pH 7.0, the serum was passed through a column of DEAE Affi-Gel Blue (Bio-Rad) equilibrated with this buffer. Protein not bound by the Affi-Gel column was applied to a gel filtration column of Sephadex G-100 Superfine in 0.02 M K_2HPO_4, pH 7.4. The single peak obtained from this column was analysed by SDS polyacrylamide gel electrophoresis and silver staining (21). Protein concentrations were determined by the method of Smith et al. (22).

Production of antibodies against axolotl transferrin

To generate polyclonal antiserum against the purified axolotl transferrin, the protein was added in phosphate buffer to Freund's complete adjuvant (Sigma Chemical Co.) at a concentration of 50 ug/ml. Six female Balb/c mice were immunized both subcutaneously in the axillae and intraperitoneally with total doses of transferrin ranging from 10 to 50 ug/ml. Two rabbits were immunized subcutaneously on their backs with 250 ug/ml. Booster injections of similar concentration were administered to the animals in the same manner two weeks later.

After an additional two weeks, antibody appearance and titer were measured by means of an enzyme-linked immunosorbent assay (ELISA). Briefly, wells of polystyrene 96-well microtiter plates were coated overnight with axolotl transferrin in solution at 2.5 ug/ml. Wells then received 100 ul of diluted antiserum or preimmune serum and were incubated 90 min. After thorough rinsing, wells received 100 ul of secondary antibody (Sigma) which was an antibody against mouse IgG (or rabbit IgG) made in goats and conjugated to alkaline phosphatase. After 60 min incubation and further washing, substrate for the enzyme was added and color development allowed to proceed for 30 min, at which time the reaction was read spectrophotometrically in a Bio-Tek EL309 microplate reader.

Two mice were also used for production of monoclonal antibodies against axolotl transferrin in the Monoclonal Facility at Indiana University. Using standard methods (23), spleen cells from the immunized mice were fused with myeloma cells and the resulting hybridomas grown and then cloned. Growth medium from these cultures was screened for presence of antibodies against axolotl transferrin by the ELISA method described above, substituting 100 ul of medium for antiserum. Medium was also tested in a second ELISA using horseradish peroxidase-conjugated secondary antibody with transferrin adsorbed to a nitrocellulose support.

Hybridomas showing high concentrations of anti-transferrin by one or both ELISA were grown to large quantities. Cells from such clones were frozen in liquid N_2 for future production of

antibody and medium from such cultures was assayed and frozen in aliquots for use in assays measuring transferrin in tissue extracts. Hybridomas were also used to produce ascitic fluid in four other mice. IgG was purified from the ascitic fluid by chromatography on Affi-Gel Blue as described above.

Immunolocalization of transferrin

Rabbit antiserum against axolotl transferrin was used for indirect immunofluorescent localization of the protein in nervous tissue. Tissue was fixed in methanol and prepared for paraffin sectioning according to the method of Sainte-Marie (24). Methanol-fixed peripheral nerves were also examined as teased wholemount preparations. Unfixed tissues were embedded in Tissue-Tek compound (Miles Scientific), frozen in liquid N_2 and sectioned on a cryostat. The secondary antibody was fluorescein isothiocyanate-conjugated goat antibody against rabbit IgG (Sigma). Localization was observed with a Nikon Fluophot epifluorescent microscope. Other nerves and ganglia were embedded in Araldite, thin sectioned and processed for observation by transmission electron microscopy by routine methods.

Enzyme-linked immunoassay for transferrin

The enzyme immunoassay developed for quantification of transferrin in this project was of a different, more efficient and sensitive design from that originally proposed in the statement of work. Development of the modified immunoassay was prompted by two factors arising early in the project. First, sufficient transferrin was purified to generate antiserum against the protein in rabbits as well as in mice. The availability of

antibodies from two species allowed greater flexibility in designing the immunoassay. Secondly, our department acquired an automated, dual wavelength Bio-Tek EL309 spectrophotometric reader for 96-well microtiter plates, which was interfaced with a microcomputer and printer. This allowed us to use 96-well plates rather than nitrocellulose in an enzyme-linked immunosorbent assay (ELISA). This made results available much more rapidly since they came directly from the plates.

ELISA's of various designs were tested for sensitivity and level of background. The best results were obtained with a noncompetitive, "sandwich"-type ELISA, similar to one described by Tijssen (23). The design of this assay is shown schematically in Figure 1. Briefly, the steps involved in the ELISA were as follows. Wells of a 96-well plate were coated with rabbit antiserum against axolotl transferrin at a dilution of 1:1500 overnight at 4° C. The plate was then washed 3 times with Tris-buffered saline containing Tween (TBS/Tween), after which wells were blocked with 5% bovine serum albumin for 1 hour and washed again 3 times with TBS/Tween. Samples of supernatants from axolotl tissue homogenates were than added to the wells in triplicate along with standard concentrations of purified axolotl transferrin, also in triplicate. Plates containing samples and standards were incubated at 4° C overnight, after which they were washed again in TBS/Tween. Then mouse antiserum against axolotl transferrin was added at a dilution of 1:2000 and incubated at room temperature for 2-3 hours. Plates were then washed 3 times in TBS/Tween. A 1:500 dilution of secondary antibody (goat anti-

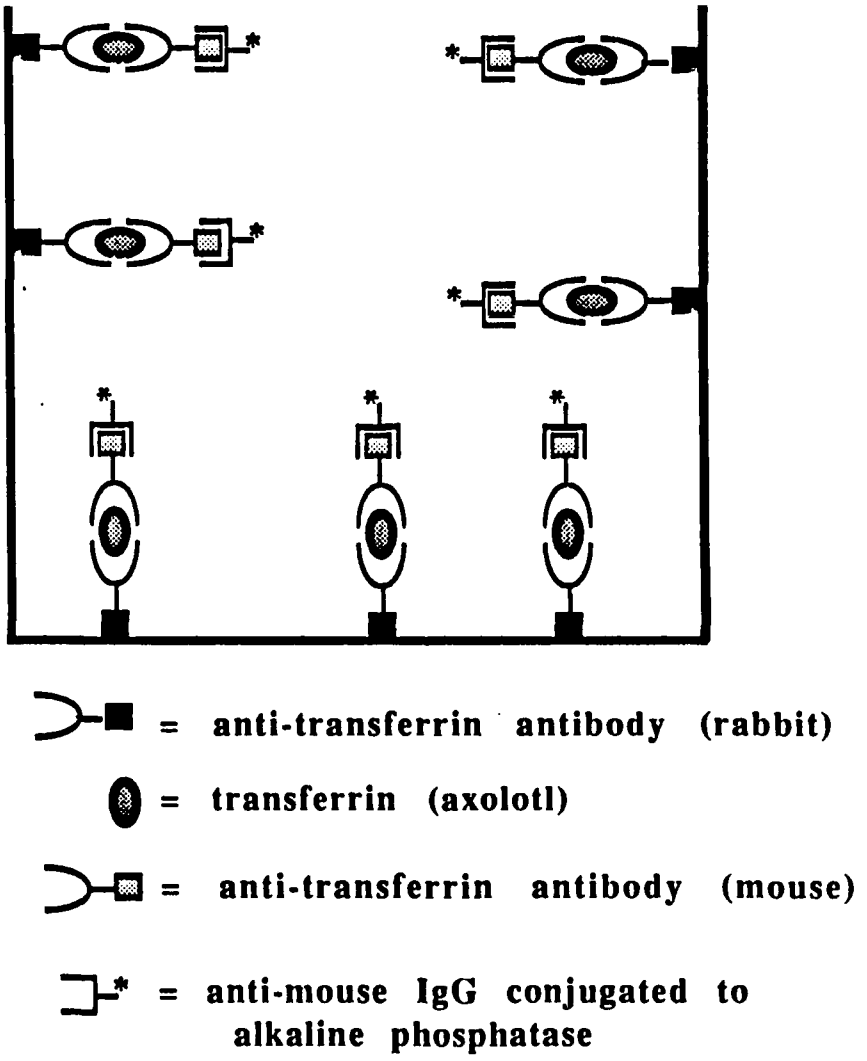

Figure 1. Design of "sandwich" enzyme-linked immunoassay (ELISA) in microtiter wells for quantitation of axolotl transferrin in tissue extracts.

mouse IgG), conjugated to alkaline phosphatase, was added to the wells and incubated 1 hour. Plates were washed 3 times with TBS/Tween. A substrate, para-nitrophenyl phosphate, was added at 1 mg/ml and incubated 30 min at room temperature. The color-generating reaction was stopped after 30 min by the addition of 3M NaOH and the plates were read at 405 nm and 540 nm with the microplate reader. Protein content of the tissue extracts was determined by the bicinchoninic acid method of Smith et al. (22).

This rabbit antibody/transferrin/mouse antibody sandwich technique with adsorption to 96-well plates provided a high level of sensitivity and low background with very simple and fast quantification of the bound transferrin. Appropriate software (Bio-Tek Instruments) was used to log the data on a floppy disk directly from the plate reader and to generate standard curves by linear regression. A typical standard curve from one experiment is shown in Figure 2.

Studies on axonal transport

To provide regenerating nerve in sufficient amounts for use in the ELISA, hindlimbs of adult axolotls (Ambystoma mexicanum), anaesthetized by immersion in 0.1% benzocaine (Sigma Chemical), were amputated unilaterally just proximal to the ankle and allowed to regenerate for various periods of time. The contralateral hindlimb was left intact as a control. Animals were maintained at 20°C on a 12 hour light/12 hour dark cycle. Limbs were used at three different periods of regeneration (early bud, palette, and digital outgrowth), which are stages in the system described by Tank et al. (25). These stages of regeneration were

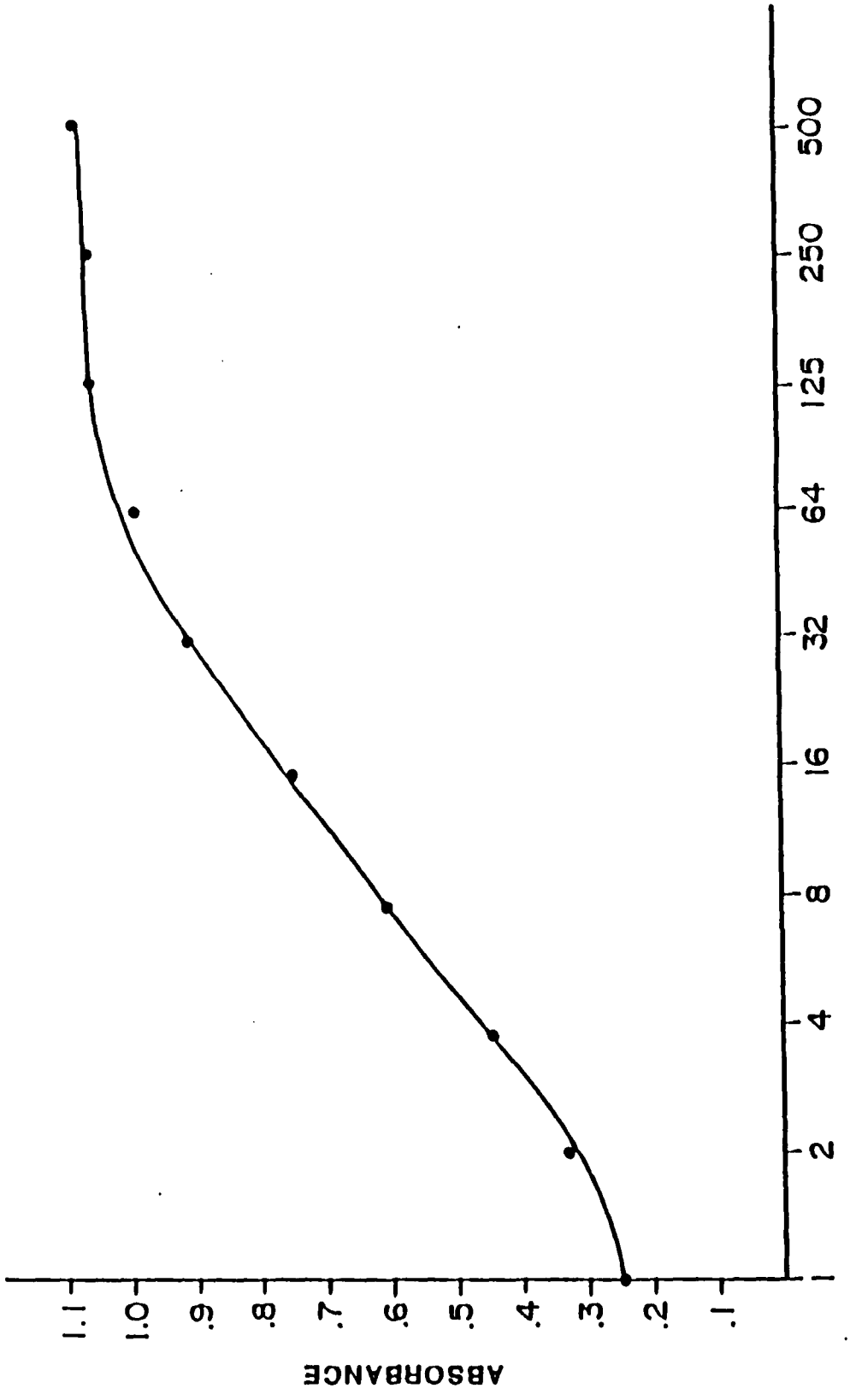

Figure 2. Typical standard curve for axolotl transferrin ELISA used in these experiments.

reached at approximately eight, ten, and twelve weeks after amputation respectively.

At the times indicated, animals were re-anaesthetized and each sciatic nerve was exposed above and below the level of the knee. Using 4-0 silk suture material, two ligatures 9 mm apart were placed on the nerve at this level, with the distal ligature at least 3 mm from the level of amputation (Figure 3). After reclosing the skin, the animals were maintained in oxygenated water at room temperature (22°C) for three hours.

At this time the animal was anaesthetized again and both sciatic nerves were removed, cleaned of investing epineurium, and cut into 3 mm segments between and on each side of the ligatures. (Animals were then sacrificed by decapitation.) Individual segments of sciatic nerve were then homogenized, using the buffer system of Meek and Adamson (26). The transferrin content of each homogenate was determined in the ELISA described above.

As a control that any transferrin accumulation obtained at the ligatures was due to axonal transport rather than to edema or vascular blockage, colchicine was prepared in a slow-release solid matrix of ethylene vinyl acetate implanted at a level just distal to the lumbar plexus. Colchicine disrupts microtubules and thus inhibits accumulation of axonally transported material at neural ligatures.

Employing standard methods for quantification of axonal transport rates in mammalian peripheral nerves (27, 28), transferrin concentrations in the segments from each nerve were used to determine the percentage of neural transferrin that was

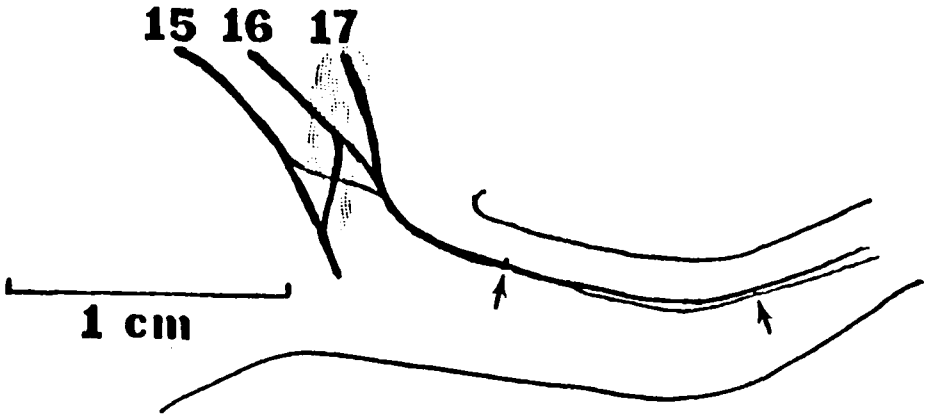

Figure 3. Adult axolotl hindlimb showing nerve supply from sacral nerves 15, 16, and 17 and locations (arrows) of ligatures placed on sciatic nerve for axoplasmic transport studies.

involved in axonal transport (the mobile fraction) and the rate at which this factor was transported in both the retrograde and anterograde directions.

Results

(1) Purification of transferrin and production of antibodies

Six preparations of axolotl transferrin totalling approximately 9 mg were purified chromatographically. The unbound fraction from Affi-Gel Blue affinity columns appeared as a single peak, which was shown electrophoretically to contain two proteins, one of which was abundant. The proteins had molecular weights of approximately 73K and 98K, as determined by comparison with markers on SDS polyacrylamide gels.

Application of this peak to Sephadex G-100 gel filtration chromatography allowed separation of the abundant protein from the contaminant. The purified protein was determined to be transferrin by the fact that its molecular weight corresponded to that of transferrin from other poikilothermic vertebrates (29, 30) and by its failure to bind Affi-Gel Blue, a characteristic property used to purify mammalian transferrin (20).

Rabbits immunized with the purified axolotl transferrin produced antisera with a titer of 50,000, as determined by ELISA. Mice immunized with the preparations of transferrin produced antisera ranging in titer from 6,500 to 100,000.

(2) Immunoassay of transferrin in regenerating limb tissue

The transferrin content of larval axolotl forelimb regeneration blastemas was measured by the ELISA method described above. As indicated in Table I, the concentration of this protein

Table I

TRANSFERRIN CONTENT OF LARVAL AXOLOTL FORELIMBS

Tissue	ng Transferrin / ug Protein (mean ± S.E.)
Unamputated Limbs (n=6)	7.48 ± 1.31
Mid-Bud Regenerate (6 Day) (n=8)	8.98 ± 3.03
6 Days Postamp., Denervated Day 5 (n=5)	4.15 ± 2.24
6 Days Postamp., Denervated Day 3 (n=5)	2.95 ± 1.07
6 Days Postamp., Denervated Day 0 (n=8)	4.25 ± 1.69

in distal regions of mid-bud stage blastemas (6 days after amputation) was not significantly different from that in complete, unamputated limbs. Surgical devascularization of limbs or mid-bud stage blastemas by transection of the brachial artery and vein had no consistent significant effect on transferrin concentration one day later (data not shown), probably due to the fact that circulation was re-established in the limb by this time. However, transection of the brachial nerves near the brachial plexus one day before the blastemas were sampled (5 days postamputation) reduced the transferrin concentration in distal tissues by approximately 50% (Table I, Figure 4). This effect of denervation was similar if nerves were transected as early as 3 days postamputation or even on the day of amputation. These observations indicate that the adverse effect of denervation on the transferrin concentration in the limb is rapid and lasts at least 6 days. The latter result is consistent with our earlier finding that denervated and amputated larval forelimbs are not reinnervated distally for 10 days (24).

Denervation of larval Ambystoma forelimbs at any early stage of regeneration leads to a rapid decrease in cell cycling and growth. It is also known that transferrin uptake is greatly increased as cells begin to proliferate. It is possible therefore that the reduced concentration of transferrin after denervation is related to reduced proliferative activity rather than to a reduction in the content of nerves. To test this possibility, larvae were X-irradiated (2000 rads) with one forelimb shielded and forelimbs were amputated bilaterally one day later. As

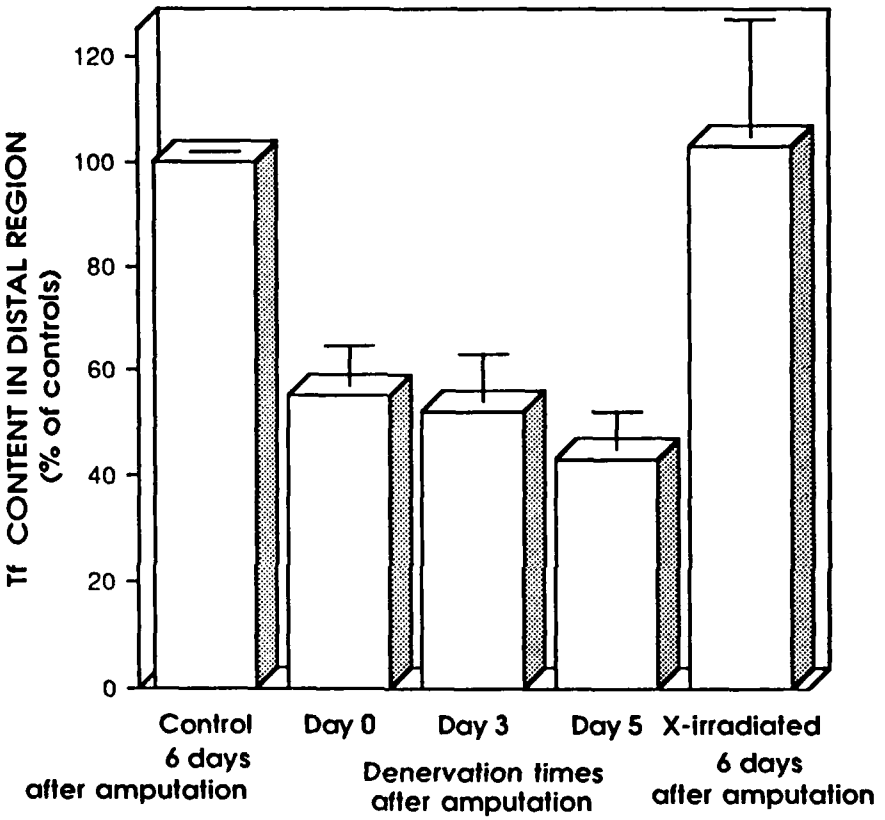

Figure 4. Effects of denervation at various times and of X-irradiation on transferrin concentration in distal region of regenerating larval axolotl forelimbs, expressed as percentage of concentration in contralateral control regenerating limbs. Bars indicate means $\pm$ S.E. of 4-6 limbs.

expected, such X-irradiated limbs failed to regenerate, while the shielded limb underwent blastema formation normally. Six days after irradiation, when the control limbs were at the mid-bud stage of regeneration, distal regions of both forelimbs were sampled. As shown in Figure 4, inhibition of growth by X-rays ha no effect on transferrin content. This control experiment strongly suggests that the 50% decrease in the transferrin content of denervated limb stumps is due to the loss of axons from the tissue, which is consistent with our hypothesis that nerves are an important source of transferrin for blastemal cells.

(3) Localization of transferrin in peripheral nerves

Rabbit antiserum against axolotl transferrin was used with a fluorescein-conjugated secondary antibody in attempts to localize this protein in teased whole-mount preparations of axolotl brachial nerve. The results showed positive staining for transferrin in both axons and the sheath of Schwann cells surrounding axons (Figure 5). Nerves stained with a similar dilution of control (pre-immune) rabbit serum were completely negative (not shown). These immunocytochemical results are consistent with those obtained by others with chick peripheral nerve (32).

As expected immunostaining of frozen sections of axolotl brachial nerve revealed the presence of transferrin in the lining of blood vessels as well as in the perineurium (data not shown). Transferrin staining in perineurium is of interest, since this tissue is a major component of the blood-nerve barrier regulating

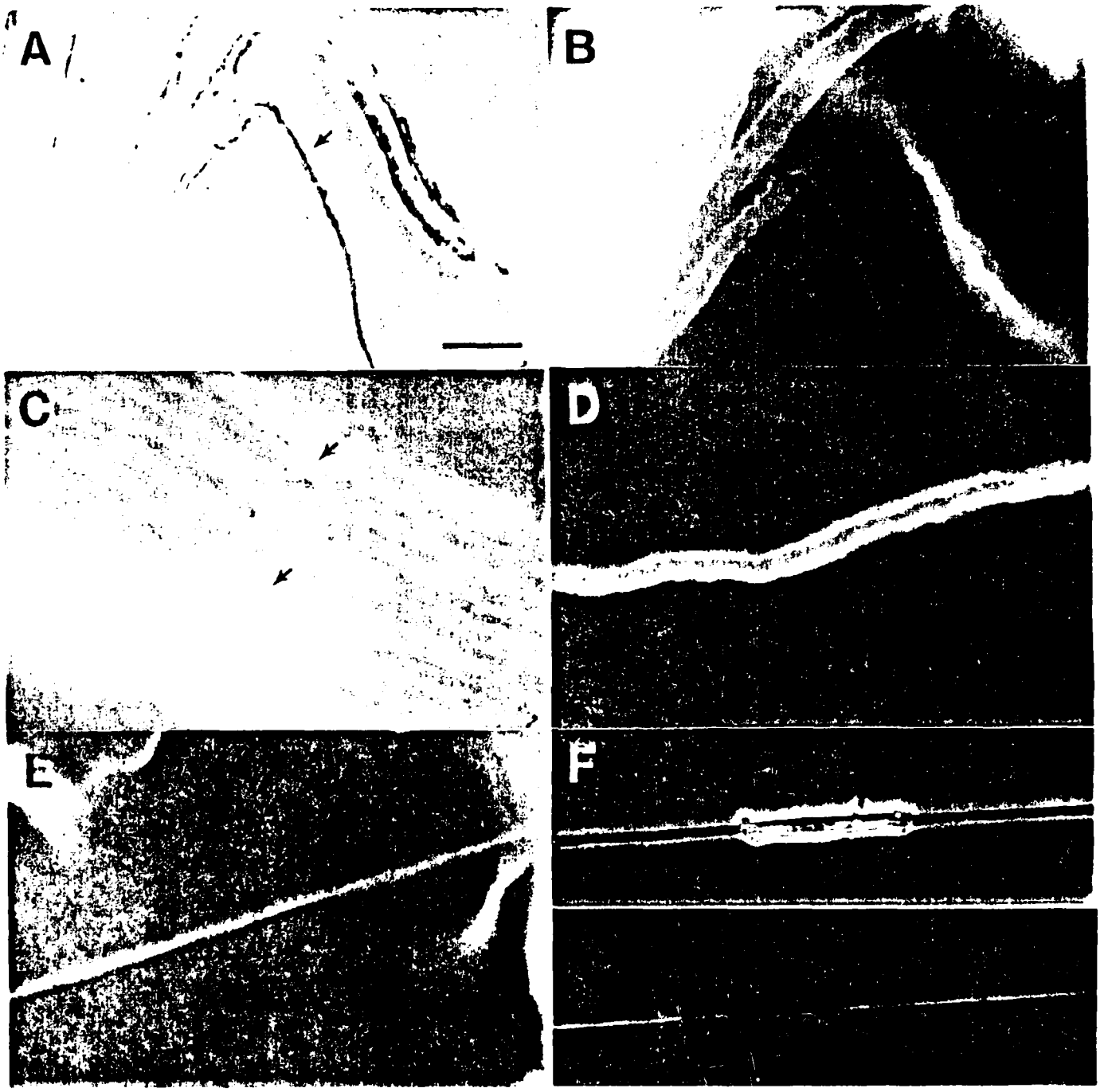

Figure 5. (A) Phase contrast of teased fibers from axolotl peripheral nerve showing one axon (arrow) from which the myelin sheath has been stripped. (B) Same field as in A, showing indirect immunofluorescence after treatment with anti-Tf antiserum. Myelin sheaths and axon are both labeled. Control preparations treated with preimmune serum were completely devoid of fluorescence (not shown). (C) A bundle of myelinated axons, showing nodes of Ranvier (arrows), after anti-Tf staining. (D) A single fiber with myelin sheath after anti-Tf staining. (E) A single fiber stripped of its myelin sheath after anti-Tf staining. As a control to confirm the identification of the bare axons, preparations of nerve fibers were also processed by indirect immunofluorescence following treatment with an antibody against neurofilament protein. (F) A single fiber partially covered by a fragment of myelin sheath, shown with phase contrast (top) and fluorescence microscopy (bottom), demonstrates labeling only in exposed regions. All x500 (bar=25um)

availability of plasma proteins and other factors to cells of the endoneurium, Schwann cells and axons. The barrier consists of flattened cell processes of the perineurial fibroblasts which are joined by tight junctions (33). Transport across the flattened cell processes is apparently due to receptor-mediated endocytosis and release on the other side. The localization of transferrin in the perineurial processes at the light microscope level may indicate that this tissue is involved in uptake of transferrin from interstitial fluid outside the nerve and delivery of this factor to the endoneurial environment. Examination of the axolotl brachial nerve perineurium by routine electrom microscopy shows that the flattened cellular processes contain numerous micropinocytotic vesicles (Figure 6), indicating extensive uptake and transport of material in these cells. However, attempts to identify transferrin in these vesicles using methods of immunolocalization by electron microscopy have to date proved unsuccessful.

(4) Immunoassay of transferrin in regenerating nerves

Using the ELISA to measure transferrin concentrations in tissue extracts, the mean concentration of transferrin in the segments of adult axolotl sciatic nerves was found to increase dramatically during the process of nerve and limb regeneration (Table II). Unilateral amputation caused the transferrin concentration in the affected sciatic nerve to increase approximately 3-fold by the early bud stage of limb regeneration, 7-fold by the palette stage, and 20-fold by the digital outgrowth stage. Somewhat smaller increases in transferrin content also

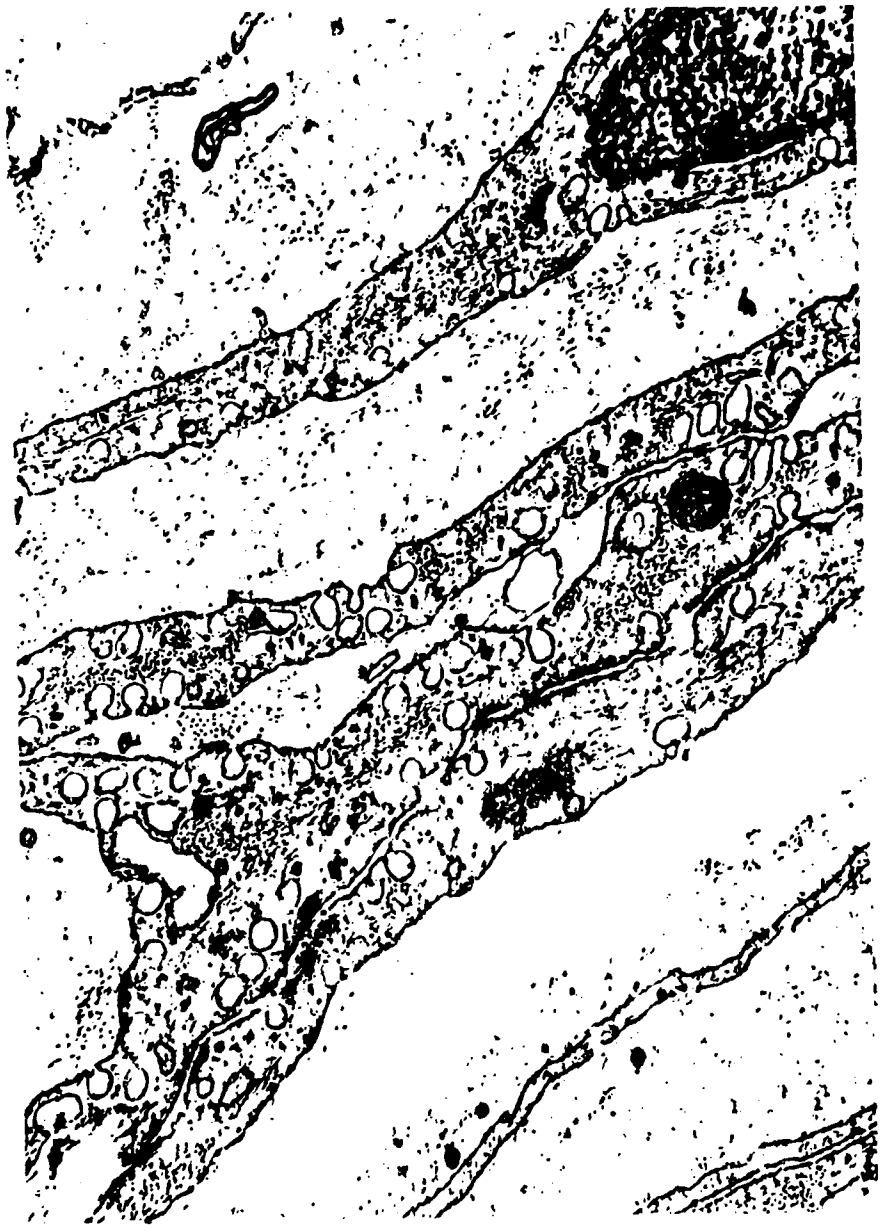

Figure 6. Electron micrograph of perineural layers adjoining epineurium (top of figure) in sciatic nerve of adult axolotl. Flattened cellular processes have numerous vesicles and invaginations on both sides suggesting transcytosis. (x20,000)

Table II

TRANSFERRIN CONCENTRATIONS (ng/mm) IN SCIATIC NERVES FROM REGENERATING AND CONTRALATERAL CONTROL LIMBS

Stage of Regeneration[1]	Regenerating	Control
(unamputated)	----	9.1 ± 1.1 (3)
early bud	28.1 ± 4.0 (4)	14.3 ± 2.4 (4)
palette	62.7 ± 11.4 (4)	54.4 ± 13.9 (4)
digital outgrowth	213.7 ± 49.4 (3)	159.3 ± 19.1 (3)

[1]Staging system of Tank et al. (1976)

[2]Mean ± S.E. (n)

occurred in the contralateral intact sciatic nerves during this period, suggesting stimulation of transferrin uptake or synthesis in these intact nerves by an unknown humoral factor produced in response to the contralateral limb or neural injury.

Transferrin concentrations in nerve segments from regenerating limbs were analyzed in relation to the ligatures. Taking the average concentration between the ligatures as 100%, the two segments proximal to the first ligature were found to contain approximately 250% of this amount (Fig. 7). This result implies that transferrin was being transported in the anterograde (proximal to distal) direction during the three hour period of ligation. No accumulation was found distal to the second ligature, suggesting little or no retrograde transport of this protein. The actual mean concentrations of transferrin in the segments proximal and distal to the ligatures from nerves at the three stages of limb regeneration examined are given in Table III.

To investigate the basis for the accumulation of transferrin at the ligature, colchicine was applied to the sciatic nerve at a level distal to the lumbar plexus using a polymer designed for the slow release of incorporated compounds. Colchicine was prepared in the polymer (Elwax) at a concentration of 10 mg/cc and a 0.5 cc implant was used for each limb. As shown in Figure 8, this treatment 3 days before ligation completely inhibited accumulation of transferrin at the ligature during the three hour period of nerve ligation. This indicates that the accumulation is due to microtubule-based axonal transport, rather

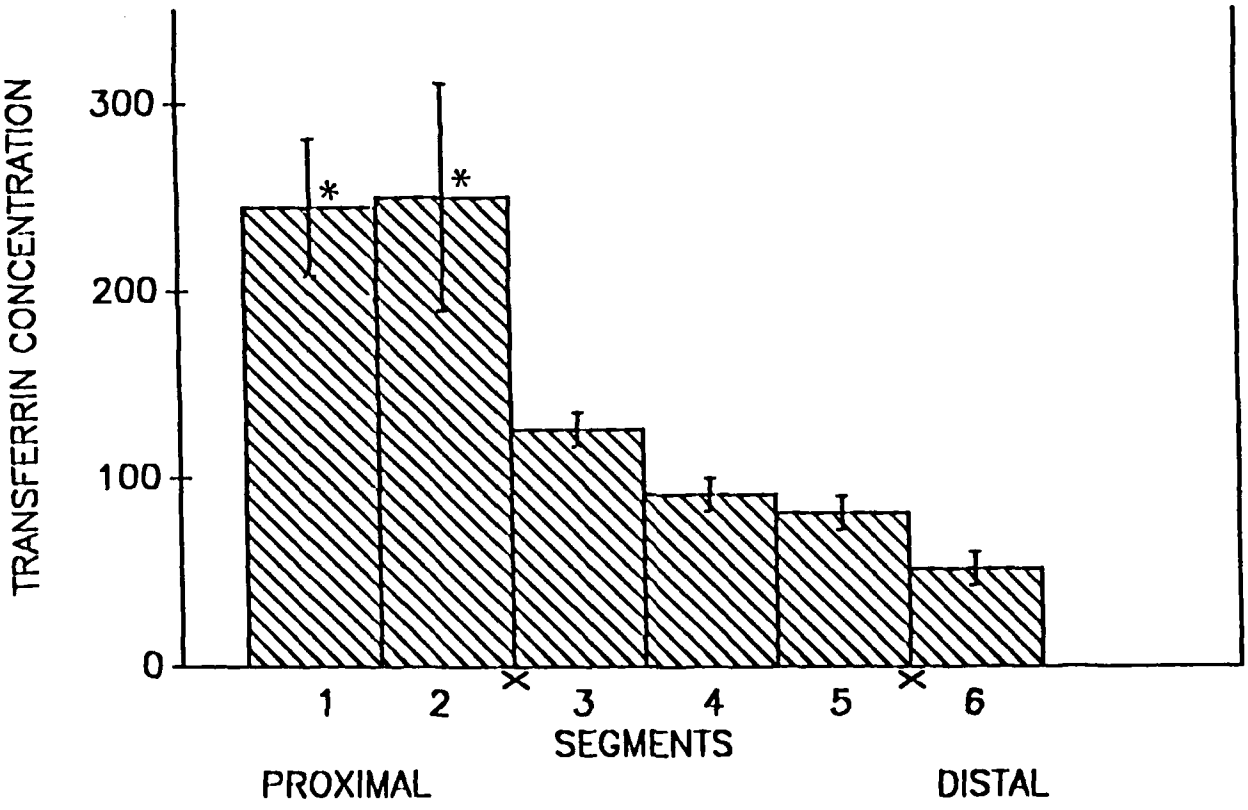

Figure 7. Transferrin concentrations in segments of regenerating sciatic nerves 3 hours after ligation as percentages of the average concentration in the three segments between the ligatures. Locations of ligatures indicated by "X's". Each bar indicates mean $\pm$ S.E. of 11 segments. Transferrin accumulates in both segments proximal to the first ligature.

Table III

AXONAL TRANSPORT OF TRANSFERRIN AT THREE STAGES OF LIMB REGENERATION[1]

Transferrin in sciatic nerve	EARLY BUD	PALETTE	DIGITAL OUTGROWTH
Average concentration (ng/mm)	28.1 ± 4.0 (4)[2]	62.7 ± 11.4 (4)	213.7 ± 49.4 (3)
Concentration proximal to ligature (ng/mm)	54.3 ± 16.9 (4)	126.8 ± 47.7 (4)	354.4 ± 73.2 (3)
Concentration distal to ligature (ng/mm)	10.2 ± 3.6 (4)	26.1 ± 13.1 (4)	----
Mobile fraction	0.41 ± 0.15 (4)	0.39 ± 0.18 (3)	0.22 ± 0.10 (3)
Anterograde transport rate (mm/d)	74.7 ± 33.9 (4)	75.8 ± 43.8 (3)	76.9 ± 27.5 (3)

1 Staging system of Tank et al. (1976)
2 Mean ± S.E. (n)

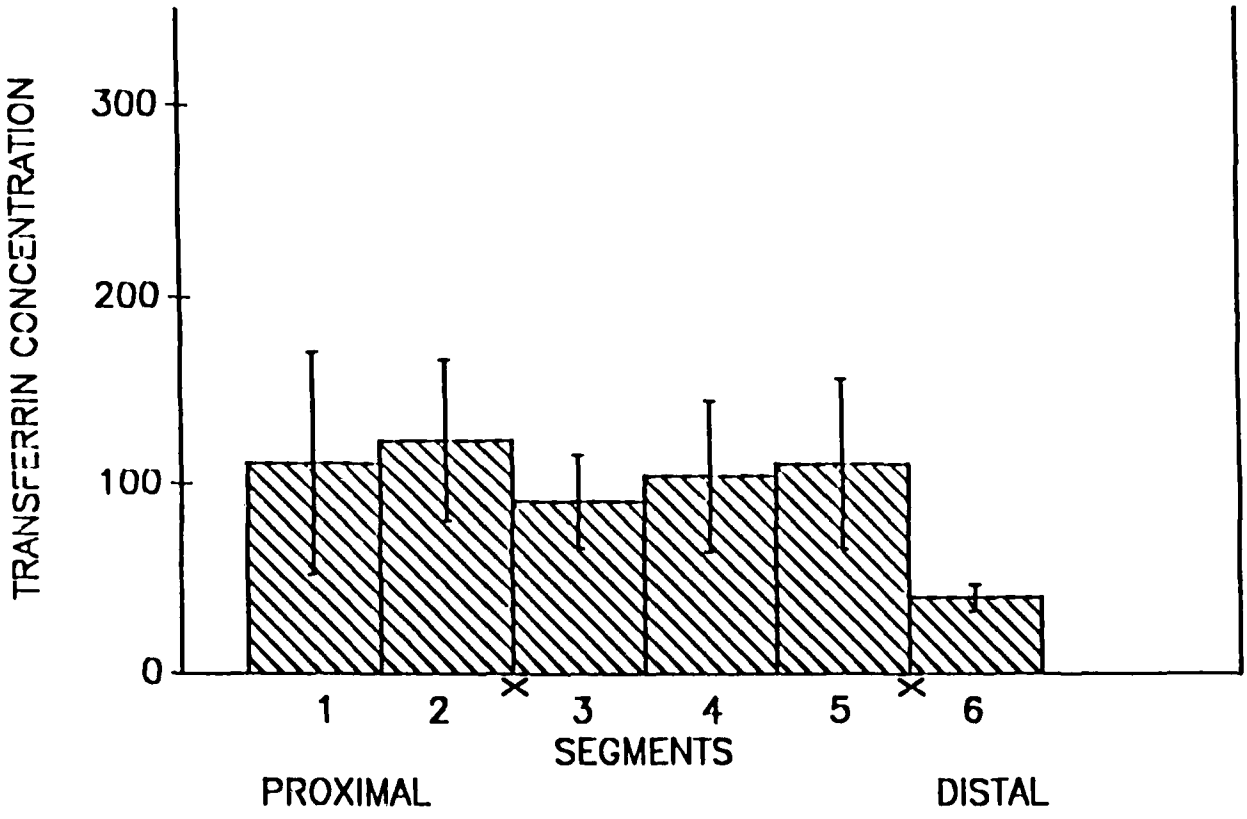

Figure 8. Transferrin concentrations of nerve segments as in Figure 7 but following local treatment of the nerves with colchicine distal to the lumbar plexus. Each bar indicates mean $\pm$ S.E. of 4 segments. Accumulation at the ligatures is abolished by colchicine indicating its dependence on axonal transport rather than edema or vascular blockage.

than vascular blockage or edema produced by the ligature. This control experiment was particularly important in this axonal transport study because the protein under investigation is an abundant plasma protein and therefore might be expected to become concentrated at sites of edema or blood accumulation.

The average transferrin concentrations proximal, distal, and between the ligatures were used to calculate the mobile fraction and transport rate of this protein in both regenerating and intact nerves. Although this concentration increased in the nerves during limb regeneration, the percentage of transferrin undergoing axonal transport in the nerves (the mobile fraction) did not change significantly during this period (Table III). The mobile fraction of this protein in control intact nerves was also not significantly lower than that of regenerating nerves (Table III).

The rate of transferrin transport in the anterograde direction was also similar in the regenerating nerves at all three stages of limb regeneration and in all intact nerves (except those in the early bud stage group). This rate averaged 70-75 mm/day or 3 mm/hour, which clearly indicates that transferrin is carried in the fast component of axoplasmic transport which involves proteins carried in vesicles.

Discussion

In the first phase of this project axolotl transferrin was purified and antibodies against this protein were produced. These were used successfully during the project's second phase to develop a rapid and sensitive ELISA for measurement of

transferrin in tissue extracts and to localize this protein in tissue sections for light microscopy. In the last phase these techniques were used in experiments that have yielded valuable new information regarding transferrin in peripheral nerves and the role it may play in developmental interactions between growing nerves and the cells of surrounding tissues.

Immunohistochemical investigations of axolotl brachial nerves with antibodies to transferrin showed the presence of this factor in both axons and the sheath of Schwann cells around the axons. These findings confirm for this amphibian similar immunofluorescent data reported for peripheral nerves of chick (32) and rat (14). The source of transferrin in axons and Schwann cells is remains unclear but evidence has been presented for synthesis of the protein in motor neurons (13) and for receptor-mediated uptake of the protein by axons and neuronal perikarya (12). Endocytosis of plasma proteins by axons would require transport of the proteins across the perineurium, the major component of the blood-nerve barrier in peripheral nerves. It is of interest therefore that we have found that the perineurium of axolotl brachial nerves also stains heavily for transferrin and that by transmission electron microscopy the flattened cellular processes of this tissue are seen to contain many vesicles indicative of transcytosis or transport of proteins across the barrier. While we have not yet been able to localize transferrin specifically to these vesicles by immunoelectron microscopy, the results are consistent with the concept that this protein is

taken up by neurons from interstitial fluid after transport across the perineurium.

The ELISA developed in this project allowed quantitative studies on transferrin contained within axolotl nerves and regenerating tissues. The concentration of transferrin in distal injured tissue in forelimbs of larval axolotls amputated through the radius/ulna and denervated by transection of all three brachial nerves at the brachial plexus was found to be approximately half that present in control, regenerating limb stumps six days after amputation or in comparable levels of intact limbs. Control experiments using local X-irradiation of the limbs rather than denervation to inhibit regenerative growth after amputation indicated that the reduced transferrin concentration was due to loss of nerves and not to the lack of proliferating cells. Temporary devascularization of limb stumps by transection of the brachial artery had no detectable effect on transferrin content of the limbs one day after the operation, but this result may be due to the extremely rapid revascularization of the limb tissue which precludes direct comparison with the denervation experiments.

The decrease in tissue transferrin content was similar if limbs were denervated at the time of amputation or if denervation was delayed until one day before tissues were sampled, indicating that the effect lasts at least six days and that it occurs rapidly, within one day. This is consistent with previous results since degeneration of axons in larval forelimbs is rapid following denervation and regrowth of such axons to the level of

amputation takes approximately ten days (4,31). The loss of transferrin in distal tissues of the limb stump following axotomy supports the hypothesis that nerves are a source of this factor for proliferating cells of the regeneration blastema.

The study reported here represents the first quantitative examination of transferrin in injured, regenerating peripheral nerves and the first study on axonal transport of any plasma protein. The observation that the concentration of transferrin increases greatly in peripheral nerves during their regeneration correlated with the requirements for rapid metabolism at growth cones of regenerating axons. The large number of mitochondria in growth cones suggests a requirement for iron needed as the cofactor in several mitochondrial respiratory enzymes. In support of this idea, a correlation has recently been shown between the accumulation of transferrin and the formation of new mitochondria in developing neurons in rats (11). The need for new mitochondria during axonal growth may dictate increased uptake of the iron-transport factor. In adult rat peripheral nerve, the onset of regeneration induced by crush injury has recently been shown to stimulate a rapid increase in the number and density of transferrin receptors on the neuronal cell bodies and this increase is followed by an accumulation of iron by the regenerating neurons (34). Our results showing increased transferrin accumulation in regenerating axolotl sciatic nerves are consistent with this report. The increased number of transferrin receptors indicates that the concentration of

transferrin in nerves may be largely due to increased uptake by the neurons.

The elevated amounts of transferrin in regenerating nerves which we have demonstrated provides a reasonable explanation for the greater growth-promoting activity of such nerves compared to that of intact nerves. Early studies by Singer (reviewed in ref. 3) showed that the motor nerve component of salamander forelimbs alone was not capable of supporting limb regeneration, but that if the motor nerves themselves were regenerating as a result of a previous injury they did have the capacity by themselves to support blastema formation and limb regeneration. This result was recently confirmed and extended by Maier et al. (35). Also, in a limb regeneration study with a completely different experimental design, it has been shown that extracts of regenerating neural tissue have greater mitogenic activity for cultured blastema cells than similar extracts of normal neural tissue (36). The basis of the increased growth-promoting activity in nerves during their own regeneration, observed both in vivo and in vitro, remains unclear. However the present results with transferrin suggest for the first time a mechanism that is not only plausible physiologically, but strongly supported by the well-known requirements of this factor for axonal growth and cell proliferation.

Why the transferrin concentration in the contralateral intact nerves was also affected by amputation is not clear. However, similar "transneuronal" effects have been reported in several other studies of neuronal response to unilateral injury.

Using another species of salamander, Tweedle (37) examined the effect of unilateral forelimb amputation on motor and sensory neuronal cell bodies of both forelimbs. Both injured and intact nerves showed similar chromatolytic reactions and increased RNA synthesis. The bilateral effect on nerves following unilateral injury suggests that the response to injury may involve release of a humoral factor that stimulates a multifaceted neuronal activation.

Because of the specialized nature of the work, our studies represent the first time axonal transport of any exogenous factor important for growth has been examined during amphibian limb regeneration. Previous work in the field of limb regeneration, reviewed by Wallace (38), suggested that the trophic factor promoting blastema growth may in fact be included in the fast transport component of axons, but this possibility remained untested prior to this study. The data obtained from the double ligature study indicate that transferrin is transported along axons in a proximal-to-distal direction and that retrograde transport of this factor, if it occurs at all, is much less important quantitatively. In two previous preliminary single ligation studies of axonal transport, by Oh and Markelonis (7) using chickens and by Tomusk and Mescher (unpublished observations) using adult rats, transferrin transport was observed in both directions. The cause of these disparities, possibly owing to differences in methodology or species, and its significance remain to be resolved. Whatever the cause, the apparent lack of retrograde transport in the axolotl does not

affect the utility of the present results in evaluating the hypothesis which forms the basis for this project.

Since transferrin is a protein very abundant in blood and lymph, the control experiment for the transport studies which involved the effect of colchicine on accumulation at the ligatures was particularly important. It is possible that ligation of a sciatic nerve could produce vascular blockage or edema at the constricted site, which would likely produce increased local concentrations of all plasma proteins. Such accumulation of plasma protein, however, would still occur in the presence of the microtubule inhibitor colchicine. The observation that colchicine blocked accumulation at the ligature strongly suggests that such accumulation is due to transport of transferrin along microtubules in axons, as in the fast component of axonal transport. This conclusion is further strengthened by the observation that accumulation occurred on one side of the ligature only; protein accumulation due to edema or vascular constriction typically occurs in both directions (39).

The distal transport of this growth-promoting factor is significant for understanding the permissive effects axons exert on proliferating blastema cells. If the quantity of axonally transported Tf that was found to accumulate at the proximal ligature was dispersed from growing axons in an early bud blastema at that level, a local concentration would be maintained within the blastema that correlates very well with the optimal Tf concentration for proliferation <u>in vitro</u> (16,18).

The rate of transferrin transport, as calculated from the ligation data, clearly puts this protein in the fast component and is similar to the rate reported in other axonal transport studies using amphibian nerves (40,41). As discussed above, such a transport rate for the growth-stimulating factor of nerves has been predicted from earlier work on the neural effect on blastema formation (38). This rate is also consistent with our finding that brachial nerve axotomy reduces the transferrin concentration in forelimb blastemas within as little as one day of the operation. Moreover, the rate of transport was similar in both intact and regenerating nerves, despite the higher concentration of neural transferrin during regeneration. This observation is consistent with other studies of axonal transport in regenerating nerves which indicate that regeneration does not affect the rate of fast transport (42).

Summary and Conclusions

Specific antibodies against axolotl transferrin have been used to develop a rapid and sensitive ELISA for measurement of this protein in tissue extracts and to localize it in axons and Schwann cells by light microscopy. The ELISA has shown that the transferrin concentration in distal regions of the regenerating axolotl limb decreases by approximately 50% following denervation. In conjunction with double ligation studies on axolotl sciatic nerves, the ELISA has been used to determine the direction, magnitude, and rate of axonal transport of transferrin in the major nerve supply to the regenerating limb. This work

represents the first study of axonal transport of a protein important for growth in regenerating amphibian limbs.

The results show clearly that neural transferrin is transported in axoplasm, in the anterograde direction, and at a rate expected of the mechanism involving proteins in vesicles transported along microtubules. Such transport of the trophic material for limb regeneration has been suggested for many years (3,38), but these studies represent the first clear demonstration of such a mechanism.

The results strongly support the hypothesis concerning a role for neural transferrin in limb regeneration. If transferrin undergoes exocytosis from axons, as it does from other cells after release of iron to cytoplasmic factors, the released protein will bind additional ferric ion and be available to meet the iron requirements of local cells. If transferrin is not available in sufficient concentration from the plasma, then a dependence on axons for this factor supporting growth may be apparent. The early, nerve-dependent limb regeneration blastema is avascular (2) which, together with the proteolytic nature of the extracellular microenvironment in the blastema (2), suggest that the supply of plasma proteins such as transferrin from capillaries is likely to be reduced locally. Transferrin released from axonal growth cones, which are abundant among the cells of the blastema (2), would supplement that available from plasma. The axonally secreted protein would bind iron released from local sources, such as degenerating erythrocytes or other cells, and

then bind receptors on blastema cells to deliver the iron needed for their for proliferation.

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Personnel with contract support and graduate degrees awarded

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William R. Kiffmeyer Ph.D. to be awarded 1991